The membrane distal half of gp130 is responsible for the formation of a ternary complex with IL-6 and the IL-6 receptor

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Abstract Gp130 is the signal transducing subunit of the interleukin-6 receptor. Signaling is initiated by the complex formation of gp130 with IL-6 bound to the IL-6 receptor (IL-6R). We have subdivided the extracellular domain of gp130 in two parts and expressed the mutant proteins as soluble IgG fusion proteins in COS-7 cells. By studying the formation of the ternary complex we show that the membrane distal half of gp130 which contains a cytokine receptor domain is responsible for the interaction with the IL-6/IL-6R complex. Interestingly this is the same region which is believed to be involved in specific recognition of the related cytokines LIF, OM, and probably also of CNTF and IL-11.

Key words: gp130; Structure–function analysis; Cytokine receptor domain; Interleukin-6

1. Introduction

Recently, IL-6, CNTF, OM, LIF and IL-11 were defined as a family of pleiotropic cytokines with overlapping biological activities. They share the glycoprotein gp130 as a common component for signal transduction across the cell membrane which may explain the functional redundancy of these cytokines [1–5].

For receptor activation, IL-6, CNTF and IL-11 first bind to a specific α-receptor subunit. Subsequently this complex associates with gp130 [6–8]. Signal transduction requires either homodimerization of gp130 as in the case of IL-6 [9], or heterodimerization with the LIF receptor as in the cases of LIF (after it has bound the LIF receptor) and CNTF [10,11]. Gp130 itself shows no measurable affinity to IL-6 [6]. Recently, however, it was shown that OM binds directly to gp130 [12,13] and that, at high concentrations, OM can inhibit the IL-6 activities on cells that do not respond to OM [13]. This is a first indication that an identical domain of the gp130 extracellular domain can interact with different cytokines/cytokine-receptor complexes.

Gp130 is a ubiquitously expressed 130 kDa glycoprotein consisting of an extracellular region of 597 amino acids, a single membrane spanning region of 22 and a cytoplasmic domain of 277 amino acids length [14]. The extracellular part of gp130 is composed of six fibronectin type III modules, the second and third of which (amino acids 125 to 320) form a cytokine receptor domain [15] characterized by four conserved cysteine residues and a WSXWS motif [16,17].

It has not as yet been determined which site(s) of gp130 is (are) involved in the complex formation with IL-6 and the IL-6R. Referring to the related growth hormone/growth hormone receptor system the structure of which has been solved [18,19] the cytokine receptor domain of gp130 can be predicted to be responsible for this interaction. We have constructed two different deletion mutants of the soluble human gp130 (shgp130) lacking either the three distal fibronectin type III modules including the cytokine receptor domain (amino acids 16 to 304) or the three membrane proximal fibronectin type III modules (amino acids 302 to 582). By studying the formation of the ternary complex between the soluble IL-6 receptor, IL-6 and these soluble gp130 deletion mutants we now show directly that the membrane distal half of gp130 comprising the cytokine receptor domain is sufficient for the interaction with IL-6 bound to its soluble IL-6 receptor.

2. Materials and methods

2.1. Chemicals

Restriction enzymes *Eco*RI, *Xho*I and *Bst*EII were obtained from Boehringer Mannheim (Mannheim, Germany) and T7 polymerase sequencing kit from Pharmacia (Freiburg, Germany). Tran[^{35}S]label was purchased from ICN (Meckenheim, Germany). DMEM was obtained from Gibco (Eggenstein, Germany) and FCS from Seromed (Berlin, Germany). Human IL-6 cDNA was a gift from Drs. T. Hirano and T. Kishimoto (Osaka, Japan). The eukaryotic expression vector pSVLgp130 was generously supplied by Dr. T. Taga (Osaka, Japan), the expression plasmid pCDM8-gp130-IgG1 was a kind gift of Dr. J. Liu (Boise, Idaho). The expression vector pSVLgp130-IgG was generated by subcloning the pCDM8-gp130-IgG1 insert into the vector pSVL. The polyclonal rabbit-anti-human IL-6R antiserum was raised against a part of the IL-6R extracellular domain [20].

2.2. Cell culture

Simian kidney cells (COS-7) were cultured in DMEM supplemented with 10% FCS, 100 mg/l streptomycin and 60 mg/l penicillin. Cells were grown at 37°C in a water-saturated atmosphere at 5% CO_2.

2.3. Construction of gp130 deletion mutants and gp130-IgG1 fusion proteins

The deletion mutants of the transmembrane gp130 were constructed using the PCR technique [21]. The mutant gp130ΔCR lacks the three membrane distal fibronectin type III modules including the cytokine receptor domain (nucleotides 112 to 978, amino acids 16 to 304). To construct this mutant we used the oligonucleotide 5′-ATCTGGTGC-TTTAGA/AACTGGAGATTCAGG-3′ (antisense) the first fifteen nucleotides of which are complementary to the gp130 nucleotides 96 to 111 upstream of the sequence to be deleted, whereas the last fifteen

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Abbreviations: CNTF, ciliary neurotrophic factor; gp130, glycoprotein 130; GM-CSF, granulocyte macrophage colony stimulating factor; IgG, immunoglobulin G; IL, interleukin; LIF, leukemia inhibitory factor; OM, oncostatin M.

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nucleotides are complementary to the downstream gp130 nucleotides 979 to 994. For a first PCR to generate the 5′ sequence of the mutant we used the expression plasmid pSVLgp130 as template. As primers we employed the described antisense oligonucleotide as well as a pSVL sequence primer 5′-GTCTTACTTCTGCTCT-3′ (sense) that anneals to the pSVL vector upstream of the gp130 cDNA. A second PCR to generate the 3′ sequence was carried out with the product received in the first PCR as sequence primer and a pSVL reverse sequence primer 5′-TCTAGTTGTGGTTTG-3′ (antisense) complementary to the pSVL sequence downstream the gp130 cDNA. Again, the template was the plasmid pSVLgp130. Analogously we proceeded with the construction of the deletion mutant gp130ΔF lacking the three membrane proximal fibronectin type III modules (nucleotides 999 to 1812; amino acids 302 to 582). The gp130 hybridizing oligonucleotide was 5′-GCACCAAG-TTTCTGG/GAATTCACTTTTACT-3′ (sense) with the nucleotides 1 to 15 hybridizing with the nucleotides 983 to 999 upstream and the nucleotides 16 to 30 annealing with the nucleotides 1813 to 1828 downstream of the gp130 sequence to be deleted. The first PCR was performed with the described oligonucleotide and the pSVL reverse sequence primer as primers and pSVLGP130 as template. For the second PCR the PCR product and the pSVL sequence primer were used as primers. All PCRs were carried out using 1 μg of the oligonucleotides described, 0.5 μg of the pSVL sequence primer or the pSVL reverse sequence primer and 1 μg of template. The PCR reactions were performed under the following conditions: first denaturation, 60 s at 94°C, annealing, 60 s at 45°C, primer extension, 60 s at 72°C, denaturation, 45 s at 94°C (35 cycles), last primer extension, 300 s at 72°C. The resulting PCR products were digested with the restriction enzymes *Xho*I and *Bst*EII and cloned into the vector pSVLgp130. The integrity of the constructs pSVLgp130ΔCR and pSVLgp130ΔF was verified by restriction analysis and by DNA sequencing.

The soluble human gp130-IgG1 fusion proteins shgp130ΔCR-IgG and shgp130ΔF-IgG, were generated by subcloning the cDNAs encoding the truncated gp130 extracellular domains into the vector pSVLshgp130-IgG [22]. The cDNAs were cut out of the vectors pSVLgp130ΔCR and pSVLgp130ΔF via the *Xho*I and the *Eco*RI restriction sites. The pSVL *Eco*RI restriction site of pSVLgp130-IgG was destroyed before.

2.4. Transfection and metabolic labeling

10^7 COS-7 cells were transiently transfected with 30 μg of plasmid DNA by electroporation (Gene Pulser, Bio-Rad). The electroporation was performed at 960 μF and 230 V. 48 hours after transfection the cells were metabolically labeled with [^{35}S]cysteine/methionine for 4 h and chased with unlabeled medium over night. Supernatant of the cells were collected and cells were lysed in 1% Nonidet P40, 10 mM Tris-HCl, pH 7.4, 0.4% Na-deoxycholat, 60 mM EDTA, 0.3% SDS, 1% Triton X-100, in the presence of the protease inhibitors PMSF (0.2 mM), aprotinine (5 μg/ml), leupeptine (1 μg/ml), pepstatin (1 μg/ml).

2.5. Formation of the ternary complex

COS-7 cells were transiently transfected with the expression vector pCDM8-huIL-6R [23,24] encoding the human IL-6R, and metabolically labeled with [^{35}S]cysteine/methionine. The generation of ^{35}S-labeled soluble human IL-6R ([^{35}S]shIL-6R) was induced by the addition of 0,1 μM PMA [23,24]. Supernatants of COS-7 cells transiently transfected with the shgp130-IgG fusion proteins were precipitated with 1.25 mg/ml protein A-Sepharose via the IgG domain at 4°C for 12 h. Complex formation was cunducted by adding 250 μl supernatant containing [^{35}S]shIL-6R and 200 ng of IL-6 to the protein A-Sepharose bound gp130 proteins to a final volume of 1 ml. After over night incubation at 4°C the IL-6/[^{35}S]shIL-6R/shgp130-IgG complexes were sedimented by centrifugation at 2000 rpm. Ternary complex formation was visualized by detection of a 55 to 60 kDa protein after SDS-PAGE (10%) and fluorography [25,26].

3. Results and discussion

The signal transducer gp130 is known to have no measurable affinity for IL-6 but specifically binds a complex of IL-6 and IL-6R [9,14]. This was not only shown for the membrane-bound receptor subunits but also for the soluble forms of IL-6R and gp130 which were found in human serum [27,28].

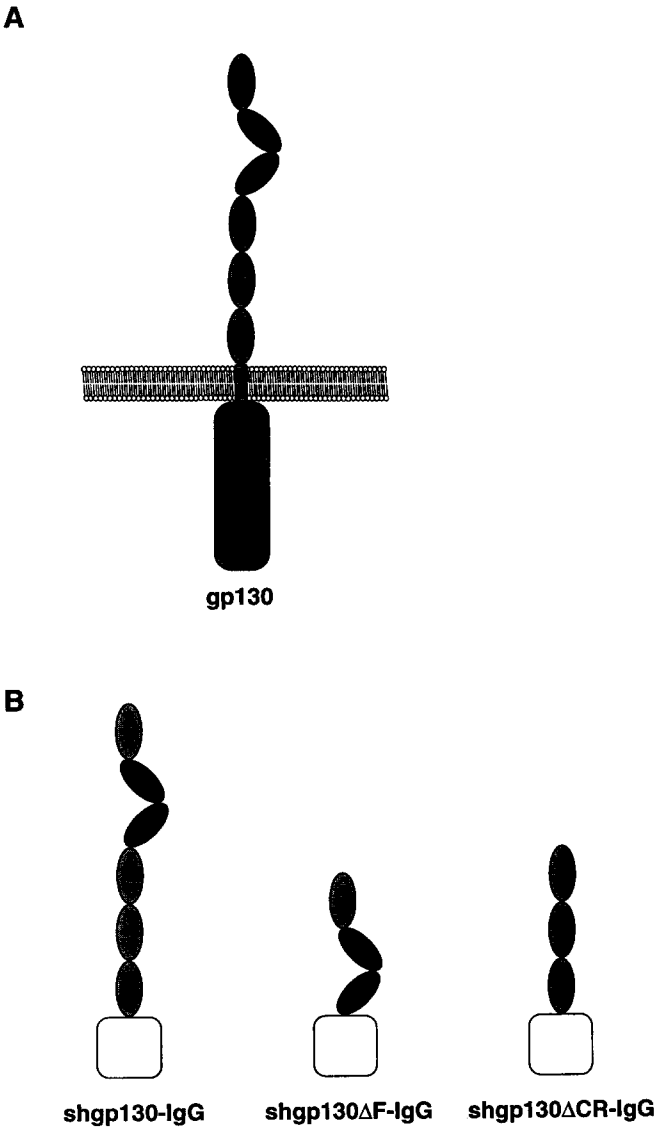


Fig. 1. Schematic representation of (A) membrane-bound gp130 and (B) IgG fusion proteins of soluble human gp130 (shgp130-IgG) and the deletion mutants shgp130ΔF-IgG and shgp130ΔCR-IgG there of. The cytokine receptor domain is shown in black, the IgG fusion protein part by the open squares.

Fig. 1A represents a schematic view of the transmembrane form of gp130. The extracellular part consists of six fibronectin type III modules, two of which form a cytokine receptor domain (shown in black). To define the gp130 extracellular region responsible for binding the IL-6/IL-6R complex, we constructed two deletion mutants of the gp130 protein and subcloned them into the expression vector pSVLgp130. To avoid the necessity of using antibodies to precipitate the ternary complex that might interfere with the complex formation, the mutant proteins were expressed as soluble IgG fusion proteins. The fusion proteins can be precipitated with protein A-Sepharose via the IgG constant domain. The truncated gp130 cDNAs were subcloned into the expression vector pSVLshgp130-IgG containing a cDNA encoding the human soluble gp130 with the constant domain of human IgG1 fused to its C-terminus (Fig. 1B). The first deletion mutant shgp130ΔF-IgG lacks the three

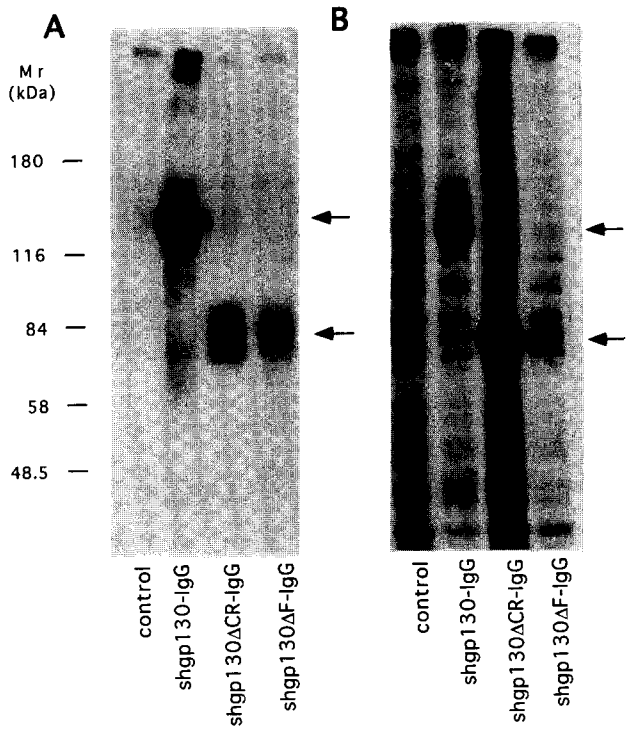


Fig. 2. Expression of shgp130-IgG, shgp130ΔCR-IgG and shgp130ΔF in COS-7 cells. 10^7 COS-7 cells were transiently transfected with 30 μg of pSVLshgp130-IgG (lane 2), pSVLshgp130ΔCR-IgG (lane 3) or pSVLshgp130ΔF-IgG (lane 4) and metabolically labeled with [^{35}S]cysteine/methionine. The ^{35}S-labeled IgG fusion proteins were precipitated from supernatants (A) and cell lysates (B) with 1.25 mg/ml protein A-Sepharose via the IgG constant domain. The proteins, indicated by arrows, were visualized by SDS-PAGE and fluorography. Non-transfected cells (lane 1) are included as control.

membrane proximal fibronectin type III modules (amino acids 302 to 582). In a second mutant shgp130ΔCR-IgG, the three N-terminal fibronectin type III modules of the gp130 extracellular domain (amino acids 16 to 304) were deleted. This deletion includes the cytokine receptor domain composed of the second and the third fibronectin repeats [14] (see Fig. 1B).

The pSVL expression vectors encoding shgp130-IgG as well as the deleted proteins shgp130ΔCR-IgG and shgp130ΔF-IgG were transiently transfected into COS-7 cells. To study their expression the IgG fusion proteins from supernatants and cell lysates of transfected COS-7 cells metabolically labeled with [^{35}S]cysteine/methionine were precipitated with protein A-Sepharose and analysed by SDS-PAGE and fluorography (Fig. 2).

The shgp130-IgG was expressed and secreted as a protein with a relative molecular weight of approximatly 120 kDa (Fig. 2A, lane 2), both the secreted deletion mutants migrated at about 75 to 80 kDa (Fig. 2A, lanes 3 and 4). The corresponding proteins detected in the cell lysates (Fig. 2B, lanes 2 to 4) are of a somewhat lower apparent molecular mass than the secreted proteins, probably due to a non-terminally processed glycosylation. It seems remarkable that although expressed at a high level, secretion of shgp130ΔCR-IgG is very inefficient (Fig. 2A and 2B, lane 3). In contrast, the only weakly expressed shgp130ΔF-IgG is exported into the medium to a larger extend (Fig. 2A and 2B, lane 4). This observation suggests that there might be a role for the N-terminal fibronectin domains in folding or secretion of the gp130 protein.

In order to investigate the ability of the gp130 deletion mutants to particpate in formation of the ternary IL-6/IL-6R/gp130 complex, shgp130-IgG, shgp130ΔCR-IgG and shgp130ΔF-IgG were expressed in COS-7 cells and precipitated from the supernatants with protein A-Sepharose. The Sepharose-bound gp130 proteins were incubated with [^{35}S]shIL-6R and human IL-6. The complexes were again sedimented, separated by SDS-PAGE and visualized by fluorography (Fig. 3).

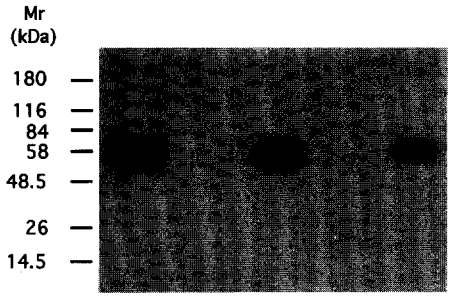
In lane 1 soluble [^{35}S]IL-6R was immunoprecipitated with a specific anti-IL-6R antiserum in the presence of IL-6. As expected, shIL-6R appears as a protein with a molecular mass of approximately 55 to 60 kDa. No radioactively labeled IL-6R was precipitated with IL-6 (lane 2) or without IL-6 (lane 3) in the absence of a specific antiserum (α-IL-6R). Lane 4 shows the co-precipitation of ^{35}S-labeled IL-6R in a ternary complex with IL-6 and shgp130-IgG. IL-6R co-precipitation did not occur when IL-6 was missing (lane 5). In lanes 6 and 7 shgp130ΔCR-IgG and shgp130ΔF-IgG were investigated. Only the addition of shgp130ΔF-IgG led to co-precipitation of the IL-6R (lane 7) indicating that only the three N-terminal fibronectin type III modules, but not the three membrane proximal modules, are required for the formation of the ternary complex.

In this first structure–function analysis of the extracellular part of the signal-transducer gp130 we have demonstrated that the three membrane distal fibronectin type III modules of the gp130 protein including a cytokine receptor domain are responsible for the formation of the ternary complex comprising IL-6 and the soluble forms of IL-6R and gp130. In analogy to the growth hormone receptor it can be hypothesized that the complex formation is brought about by the cytokine receptor domain.

Recently, it has been shown that OM binds directly to gp130 and behaves, at high doses, as an IL-6 antagonist [13]. It is interesting that apparently the same region of the gp130 cytokine receptor domain interacts with OM, with LIF (after it has bound to the LIF receptor), with IL-6, CNTF and IL-11 after they have bound to their α-receptors. For the growth hormone receptor two overlapping binding sites consisting of largely the same amino acids and with similar structures were identified to be essentiell for the recognition of two distinct sites on the growth hormone [18,19]. For the common β-chain of the human GM-CSF, IL-3 and IL-5 receptors three amino acids were found to be involved in high affinity binding of GM-CSF and IL-5 but not of IL-3 [29]. Thus, we propose that different amino acids within the same region of the gp130 cytokine receptor domain might function as binding determinants for the different cytokines/cytokine-α-receptor complexes.

As to be seen from Fig. 1A binding of the IL-6/IL-6R complex to the transmembrane gp130 cytokine receptor domain raises a distance problem. The extracellular part of the IL-6R consists of only a membrane proximal cytokine receptor domain and an N-terminal IgG-like domain. It is not clear to date how the cytokine receptor domains of IL-6R and gp130 can simultaneously make contact with IL-6 being separated by three fibronectin modules.

Further analysis of the membrane distal half of the gp130 protein should help to define more precisely where the contact with IL-6 and/or the IL-6R is made. In addition, it will be interesting to test whether a mutant of gp130 containing only



-	-	-	+	+	-	-	shgp130-IgG
-	-	-	-	-	+	-	shgp130ΔCR-IgG
-	-	-	-	-	-	+	shgp130ΔF-IgG
+	+	+	+	+	+	+	[^{35}S]shIL-6R
+	+	-	+	-	+	+	IL-6
+	-	-	-	-	-	-	α-IL-6R

Fig. 3. Formation of the ternary complex of IL-6, shIL-6R and shgp130-IgG fusion proteins. Shgp130-IgG fusion proteins were precipitated from supernatants of transiently transfected COS-7 cells with protein A-Sepharose. Complex formation was conducted by incubation of IL-6 and [^{35}S] labeled shIL-6R with the protein A-Sepharose bound gp130 proteins. The IL-6/[^{35}S]shIL-6R/shgp130-IgG complexes were sedimented by centrifugation, separated by SDS-PAGE and visualized by fluorography. α-IL-6R, monospecific anti-human IL-6R antiserum.

the cytokine receptor domain is capable of transducing an IL-6 specific signal.

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